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Mating System for Transfer of Plasmids Among *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*

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To facilitate the analysis of genetic determinants carried by large resident plasmids of *Bacillus anthracis*, a mating system was developed which promotes plasmid transfer among strains of *B. anthracis*, *B. cereus*, and *B. thuringiensis*. Transfer of the selectable tetracycline resistance plasmid pBC16 and other plasmids from *B. thuringiensis* to *B. anthracis* and *B. cereus* recipients occurred during mixed incubation in broth. Two plasmids, pXO11 and pXO12, found in *B. thuringiensis* were responsible for plasmid mobilization. *B. anthracis* and *B. cereus* transcipients inheriting either pXO11 or pXO12 were, in turn, effective donors. Transcipients harboring pXO12 were more efficient donors than those harboring pXO11; transfer frequencies ranged from 10^{-4} to 10^{-1} and from 10^{-8} to 10^{-5}, respectively. Cell-to-cell contact was necessary for plasmid transfer, and the addition of DNase had no effect. The high frequencies of transfer, along with the fact that cell-free filtrates of donor cultures were ineffective, suggested that transfer was not phage mediated. *B. anthracis* and *B. cereus* transcipients which inherited pXO12 also acquired the ability to produce parasporal crystals (Cry^+) resembling those produced by *B. thuringiensis*, indicating that pXO12 carries a gene(s) involved in crystal formation. Transcipients which inherited pXO11 were Cry^-. This mating system provides an efficient method for interspecies transfer of a large range of *Bacillus* plasmids by a conjugation-like process.

A previous report from this laboratory (15) demonstrated the utility of the generalized transducing bacteriophage CP-51 in transferring plasmids among the three species *Bacillus anthracis*, *B. cereus*, and *B. thuringiensis*. However, the size of plasmids that can be transferred by CP-51 is limited by the size of the phage and the corresponding amount of DNA it can package (ca. 50 megadaltons). Therefore, with the hope of being able to transfer the large plasmids of *B. anthracis* to assess more adequately their biological significance, we decided to investigate whether the *B. thuringiensis* mating system described by Gonzalez et al. (5, 6) could be applied to *B. anthracis*. Our strategy was to look for transfer of the selectable tetracycline resistance plasmid pBC16 (2) and then to examine tetracycline-resistant transcipients for the acquisition of additional plasmids. We have found that certain plasmids which promote their own transfer from *B. thuringiensis* are also effective in promoting the transfer of a variety of plasmids among the three *Bacillus* species.

Evidence is presented that each of two plasmids, pXO11 and pXO12, found in *B. thuringiensis* subsp. *thuringiensis* is capable of bringing about its own transfer as well as that of other plasmids. Plasmid analyses confirmed the transfer of a variety of plasmids from *B. thuringiensis* subsp. *thuringiensis* to *B. anthracis* and *B. cereus*. Transcipients of the latter two organisms that inherited either pXO11 or pXO12 were, in turn, effective donors. The mating system is thus a useful and efficient means of transferring both large and small plasmids among the three species.

MATERIALS AND METHODS

Bacterial strains. The strains of *B. anthracis*, *B. cereus*, and *B. thuringiensis* used in this study and their relevant characteristics are listed in Table 1.

Media. L broth, NBY broth, and peptone diluent were prepared as previously described (18). BHI broth contained 37 g of brain-heart infusion (Difco Laboratories, Detroit, Mich.) per liter. Min IC medium was composed of the following (in grams per liter): $(NH_4)_2SO_4$, 2; KH_2PO_4, 6; K_2HPO_4, 14; $MgSO_4 \cdot 7H_2O$, 0.2; $FeCl_3 \cdot 6H_2O$, 0.04; $MnSO_4 \cdot H_2O$, 0.00025; trisodium citrate $\cdot 2H_2O$, 1; thiamine hydrochloride, 0.01; L-glutamic acid, 2; vitamin-free acid-hydrolyzed casein (Nutritional Biochemicals Corp., Cleveland, Ohio), 5; and glucose, 5. The pH was adjusted to 7.0 with NaOH. Min IC medium was supplemented as appropriate with the required amino acids, purines, or pyrimidines at a concentration of 40 μg/ml. Streptomycin (200 μg/ml) and tetracycline (5 or 25 μg/ml) were used as indicated. For solid medium, 15 g of agar was added per liter.

Mating conditions. Donor and recipient cells were grown in 250-ml Erlenmeyer flasks containing 25 ml of BHI broth and incubated at 30°C with slow shaking. Donor and recipient strains were grown separately for 8 to 10 h from 1% (vol/vol) transfers of 14- to 15-h-old cultures. Each culture was diluted 1:50 in BHI broth, yielding 10^6 to 10^7 cells per ml, and mating mixtures were prepared by mixing 1 ml of donor cells with 1 ml of recipient cells in 20-mm culture tubes. Control tubes contained 1 ml of BHI broth and 1 ml of donor or recipient cells. Mixtures were incubated at 30°C with slow shaking. Samples were removed at the times indicated and plated on appropriate selective media for determining the number of donors, recipients, and transcipients. Dilutions were made in peptone diluent. Plates were incubated at 30°C, and colonies were scored after 24 to 48 h.

When mating mixtures were prepared with streptomycin-resistant (Str^r) recipients and tetracycline-resistant (Tc^r) donors, tetracycline-resistant transcipients were selected on L agar containing both antibiotics. If the recipients were streptomycin sensitive, tetracycline-resistant transcipients were selected on Min IC agar supplemented with tetracycline and the appropriate growth requirement of the auxotrophic recipient. For selecting *B. cereus* transcipients, 25 μg of tetracycline per ml was used, but with *B. anthracis* the

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TABLE 1. Strains used in this study

Strain[a]	Relevant characteristics[b]	Relevant plasmid(s)	Origin or reference
B. anthracis			
Weybridge	Avirulent	pXO1[c]	MRE[d]
Weybridge A	Colonial variant of Weybridge	pXO1	C. B. Thorne
Weybridge A UM17	Ade$^-$	pXO1	UV[e] of Weybridge A
Weybridge A UM17 tr57B-6	Ade$^-$ Tc^r Cry$^+$	pXO1, pXO12, pBC16	This study
Weybridge A UM23	Ura$^-$	pXO1	UV of Weybridge A
Weybridge A UM23C2	Ura$^-$, cured of pXO1	None	C. B. Thorne
Weybridge A UM23C2 tr45B-12	Ura$^-$ Tc^r Cry$^-$, cured of pXO1	pXO11, pBC16	This study
Weybridge A UM23C2 tr60B-1	Ura$^-$ Tc^r Cry$^+$, cured of pXO1	pXO12, pBC16	This study
Weybridge A UM23C2 tr96B-3	Ura$^-$ Tc^r Cry$^-$, cured of pXO1	pXO11, pBC16	This study
Weybridge A UM23C2 tr237-10	Ura$^-$ Tc^r Cry$^+$, cured of pXO1	pXO12, pBC16	This study
Weybridge UM44	Ind$^-$	pXO1	UV of Weybridge
Weybridge UM44-1	Ind$^-$ Str^r	pXO1	UV of UM44
Weybridge UM44-1 tr203-1	Ind$^-$ Str^r Tc^r Cry$^+$	pXO1, pXO12, pBC16	This study
Weybridge UM44-1 tr203-7	Ind$^-$ Str^r Tc^r Cry$^+$	pXO1, pXO12, pBC16	This study
Weybridge UM44-1 tr203-23	Ind$^-$ Str^r Tc^r Cry$^+$	pXO1, pXO12, pBC16	This study
Weybridge UM44-1 tr203-28	Ind$^-$ Str^r Tc^r Cry$^-$	pXO1, pXO11, pBC16	This study
Weybridge UM44-1 tr84-6	Ind$^-$ Str^r Tc^r Cry$^-$	pXO1, pXO11, pBC16	This study
Weybridge UM44-1 tr84-7	Ind$^-$ Str^r Tc^r Cry$^-$	pXO1, pXO11, pBC16	This study
B. cereus			
569	Wild type		NRRL[f]
569 UM20	Ant$^-$		UV of 569
569 UM20-1	Ant$^-$ Str^r		UV of UM20
569 UM20-1 tr2B-1	Ant$^-$ Str^r Tc^r Cry$^-$	pXO11, pBC16	This study
569 UM20-1 tr2B-3	Ant$-$ Str^r Tc^r Cry$^-$	pXO11, pBC16	This study
569 UM20-1 tr2B-4	Ant$-$ Str^r Tc^r Cry$^-$	pXO11, pBC16	This study
569 UM20-1 tr195B-35	Ant$-$ Str^r Tc^r Cry$^+$	pXO12, pBC16	This study
569 UM20-1 tr210B-1	Ant$-$ Str^r Tc^r Cry$^-$	pXO11, pBC16	This study
569 UM20-1 tr251-1	Ant$-$ Str^r Tc^r Cry$^+$	pXO12, pBC16	This study
569 UM20-1 tr251-5	Ant$-$ Str^r Tc^r Cry$^-$	pBC16	This study
B. thuringiensis			
4042A	subsp. *thuringiensis*	pXO11, pXO12	1
4042A UM8	Ade$^-$ Cry$^+$	pXO11, pXO12	UV of 4042A
4042A UM8 td2	Ade$^-$ Cry$^+$ Tc^r	pXO11, pXO12, pBC16	C. B. Thorne
4042A UM8-13	Ade$^-$ Cry$^-$ Osp	pXO11, pXO12	14[g]
4042A UM8-13 td1	Ade$^-$ Cry$^-$ Osp Tc^r	pXO11, pXO12, pBC16	C. B. Thorne[g]
4042A UM8-13 td1-A	Ade$^-$ Cry$^-$ Osp Tc^r	pXO11, (pXO12)$^-$, pBC16	C. B. Thorne[g]
4042B	subsp. *aizawai*		1
4042B UM45	subsp. *aizawai* Trp$^-$		UV of 4042B
4043	subsp. *dendrolimus*		NRRL
4049	subsp. *morrisoni*		NRRL
4050	subsp. *tolworthi*		NRRL
4059	subsp. *toumanoffi*		NRRL
13367	subspecies not known		ATCC[h]
33740	subspecies not known		ATCC
BTI	subsp. *israelensis*		M. deBarjac
HD-1	subsp. *kurstaki*		A. Yousten
YAL	subsp. *alesti*		A. Yousten

[a] In these strain designations, tr in the second term denotes a transcipient strain and td denotes a Tc^r transductant obtained by phage CP-51-mediated transfer of pBC16.

[b] Abbreviations: Ade, adenine; Ant, anthranilic acid; Ura, uracil; Ind, indole; Cry, synthesis of parasporal crystals; Osp, oligosporogenous; Str^r, streptomycin resistant; Tc^r, pBC16-encoded tetracycline resistance.

[c] pXO1 carries genes for the synthesis of anthrax toxin. See Thorne (in press) for a discussion of this plasmid.

[d] MRE, Microbiological Research Establishment, Porton, England.

[e] UV, Mutagenesis by UV light (14).

[f] NRRL, Agricultural Research Service, Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Ill.

[g] Although strain 4042A UM8-13 was Osp and Cry$^-$, it contained pXO12 and could be converted to Spo$^+$ Cry$^+$ by phage TP-13 (14). Strain 4042A UM8-13 td1, a Tc^r transductant of UM8-13, could also be converted to Spo$^+$ Cry$^+$. After frequent transfers of strain UM8-13 td1 on L agar slants, it was found to have lost pXO12 and consequently could not be converted to Cry$^+$ by TP-13. The (pXO12)$^-$ derivative is designated UM8-13 td1-A. Freeze-dried preparations of strain UM8-13 td1 retained pXO12.

[h] ATCC, American Type Culture Collection.

number of transcipients recovered was greater when the concentration of tetracycline was only 5 μg/ml. When transcipients were selected with the lower concentration of tetracycline, they were fully resistant to 25 μg/ml. Transfer frequency is expressed as the number of transcipients per milliliter divided by the number of donors per milliliter at the time of sampling. It should be emphasized that the use of both auxotrophic and drug-resistant strains allowed unambiguous strain selection and recognition.

Test for effect of DNase. Donor cells were first incubated alone in the presence of 100 μg of DNase per ml (Worthington Diagnostics, Freehold, N.J.) and 0.01 M $MgSO_4$ for 15 min at 37°C. Donor and recipient cells (1 ml of each) were mixed together, and DNase (100 μg/ml) was added again

after 1, 2, and 3 h of mating. $MgSO_4$ without DNase was added to control mating mixtures. After 4 h of incubation, samples were plated for selection of transcipients.

Test for effect of donor filtrates. To investigate the possibility of phage-mediated plasmid transfer, cell-free filtrates of donor cultures were substituted for donor cells. The supernatant fluid from a centrifuged donor culture was filtered through an HA membrane filter (pore size, 0.45 μm; Millipore Corp., Bedford, Mass.), and 1 ml of cell-free filtrate was mixed with 1 ml of recipient cells. Such mixtures were incubated and assayed for Tc^r transcipients as described above.

Detection of plasmid DNA. Plasmid DNA was extracted by a modification of the procedure described by Kado and Liu (10). Cells for plasmid extraction were grown in 250-ml Erlenmeyer flasks containing 25 ml of BHI broth supplemented when appropriate with tetracycline (10 μg/ml). Cultures were incubated for 16 h at 37°C on a rotary shaker (100 to 160 rpm). Cells from 25 ml of culture were collected by centrifugation at 10,000 rpm in a Sorvall SS34 rotor for 10 min at 15°C and suspended in 1 ml of E buffer (0.04 M Tris-hydroxide [Sigma Chemical Co., St. Louis, Mo.], 0.002 M EDTA [tetrasodium salt; Sigma], 15% sucrose, pH 7.9) by gentle vortexing. Cells were lysed by adding 1 ml of the suspension to 2 ml of lysis buffer, prepared by adding 3 g of sodium dodecyl sulfate and 5 ml of 3 N NaOH to 100 ml of 15% (wt/vol) sucrose in 0.05 M Tris-hydroxide. The tubes were rapidly inverted 20 times to mix the cells and buffer and were then held in a 60°C water bath for 30 min. Pronase (0.5 ml; Calbiochem-Behring, La Jolla, Calif.) solution (2 mg/ml in 2 M Tris, pH 7.0) was added, and the tubes were mixed as described above and incubated in a 37°C water bath for 20 min. The lysate was extracted with 6 ml of phenol-chloroform (1:1, vol/vol) by inverting the tubes 40 times. The emulsions were separated by centrifugation at 10,000 rpm for 10 min at 15°C, and the aqueous phase was removed for electrophoresis.

Extracts (40 μl) were mixed with 10 μl of tracking dye (25% bromphenol blue, 15% Ficoll), and samples (40 μl) were applied to horizontal 0.7% agarose (type II medium EEO, Sigma) gels prepared and run in Tris-borate buffer (0.89 M Tris-hydroxide, 0.089 M boric acid, 0.0025 M EDTA, pH 8.2 to 8.3). Electrophoresis was carried out at 70 V for 90 to 120 min at room temperature. Gels were stained with ethidium bromide (1 μg/ml in Tris-borate buffer).

RESULTS

Survey of *B. thuringiensis* strains for effective donors. We introduced the tetracycline resistance plasmid pBC16 (2) into *B. thuringiensis* strains by transduction with phage CP-51 (15) and then tested them for the ability to transfer pBC16 to *B. cereus* 569 and *B. anthracis*. A total of 12 strains of *B. thuringiensis*, representing 11 subspecies, were tested; 6 of them were effective donors, and the others were completely ineffective or very poor donors (Table 2). In these tests the *B. cereus* recipients were anthranilic acid auxotrophs and the *B. anthracis* recipients were indole auxotrophs. All of 150 or more transcipients tested retained the respective auxotrophic marker and thus could be positively identified. In addition, plasmid analysis of at least 12 transcipients from each mating confirmed the presence of pBC16 as well as a variety of other plasmids.

There appears to be some specificity involved among the various donors (Table 2). Strains 4059 (*B. thuringiensis* subsp. *toumanoffi*) and 4049 (*B. thuringiensis* subsp. *morrisoni*) were each about equally effective with *B. anthracis* and *B. cereus*. Strains YAL (*B. thuringiensis* subsp. *alesti*) and BTI (*B. thuringiensis* subsp. *israelensis*) were considerably better donors with *B. cereus* than with *B. anthracis*. The first two strains listed in Table 2 are both mutants of strain 4042A (*B. thuringiensis* subsp. *thuringiensis*). Strain UM8 is an adenine auxotroph, and strain UM8-13 is an Osp mutant derived from UM8. (As pointed out in Table 1, transductant UM8-13 td1-A spontaneously lost pXO12). The Osp mutant was considerably more effective as a donor in matings with *B. cereus* than with *B. anthracis*.

TABLE 2. Test of various strains of *B. thuringiensis* as donors of pBC16 in matings with *B. cereus* and *B. anthracis*[a]

Donor *B. thuringiensis* strain[b]	No. of Tc^r transcipients per ml with recipient strain:	
	B. cereus 569 UM20-1 Str^r	*B. anthracis* Weybridge UM44-1 Str^r
4042A UM8 td2(pBC16)	8.9×10^4	1.6×10^5
4042A UM8-13 td1-A(pBC16)	3.2×10^6	8.3×10^4
4042B UM45(pBC16)	1.0×10^2	0
4043(pBC16)	0	0
4049(pBC16)	3.0×10^4	1.7×10^4
4050(pBC16)	1.5×10^1	0
4059(pBC16)	6.4×10^3	1.1×10^4
13367(pBC16)	0	0
33740(pBC16)	8.0×10^1	0
BTI(pBC16)	1.2×10^4	4.4×10^2
HD-1(pBC16)	1.0×10^2	0
YAL(pBC16)	4.0×10^5	6.0×10^1

[a] Mating mixtures were incubated for 20 h, and transcipients were selected on L agar containing 200 μg of streptomycin and 10 μg of tetracycline per ml. Control tubes in which each strain was incubated with 1 ml of BHI broth yielded no spontaneous Tc^r Str^r colonies. The numbers of transcipients are averages of results from three experiments.

[b] See Table 1 for subspecies and characteristics.

Because we recognized early in our investigation of the mating system that there appeared to be two different fertility plasmids in strain 4042A, we chose to use that strain for further detailed study.

Transfer of pBC16 from *B. thuringiensis* subsp. *thuringiensis*. Plasmid profiles of the two donor strains 4042A UM8 td2 and 4042A UM8-13 td1-A, as well as of *B. anthracis* and *B. cereus* recipients and transcipients, are shown in Fig. 1 and 2. All Tc^r transcipients inherited plasmid DNA which migrated at the same rate as pBC16. In addition to pBC16, most of the transcipients also inherited various combinations of other plasmids derived from the *B. thuringiensis* subsp. *thuringiensis* donor. Examination of a large number of transcipients has shown that (i) the two most frequently transferred *B. thuringiensis* subsp. *thuringiensis* plasmids were pXO11 and pXO12 and (ii) the lower-molecular-weight plasmids were transferred more or less at random, as demonstrated by their variable distribution in the transcipients. In matings with donor strain 4042A UM8-13 td1-A, which harbors pXO11 as well as several other plasmids, the majority (80% or more) of the Tc^r transcipients acquired pXO11. Similarly, with donor cells of strain 4042A UM8 td2, which harbors both pXO11 and pXO12, the majority of the Tc^r transcipients inherited either pXO11 or pXO12. However, we have observed that in matings with the latter donor, transcipients inherited pXO11 more frequently than pXO12. No transcipients thus far examined have contained both of these plasmids, suggesting that there may be competition between pXO11 and pXO12 during transfer. All transcipients retained both the auxotrophic and streptomycin resist-

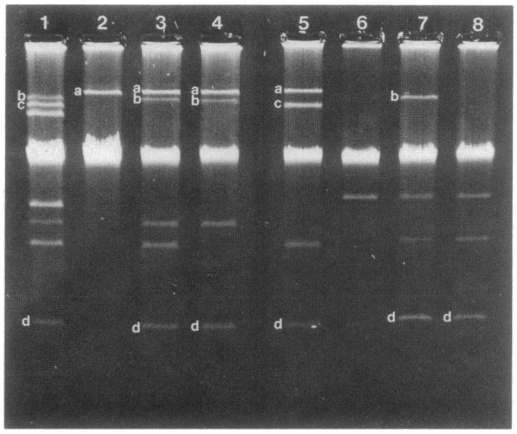

FIG. 1. Agarose gel electrophoresis of plasmid DNA from a *B. thuringiensis* subsp. *thuringiensis* donor and *B. anthracis* and *B. cereus* recipients and transcipients. Plasmid bands: a, pXO1; b, pXO12; c, pXO11; and d, pBC16. The large diffuse band in all lanes is chromosomal DNA. The molecular mass of pXO1 is 114 megadaltons as determined by restriction analysis (N. J. Robillard, Ph.D. dissertation, University of Massachusetts, Amherst, 1984) and that of pBC16 is 2.8 megadaltons. The sizes of pXO11 and pXO12 have not been determined. Lanes: 1, *B. thuringiensis* subsp. *thuringiensis* 4042A UM8 td2, Cry^+ donor; 2, *B. anthracis* Weybridge UM44-1, recipient; 3, *B. anthracis* Weybridge UM44-1 tr203-1, Cry^+ transcipient; 4, *B. anthracis* Weybridge UM44-1 tr203-7, Cry^+ transcipient; 5, *B. anthracis* Weybridge UM44-1 tr203-28, Cry^- transcipient; 6, *B. cereus* 569 UM20-1, recipient; 7, *B. cereus* 569 UM20-1 tr251-1, Cry^+ transcipient; 8, *B. cereus* 569 UM20-1 tr251-5, Cry^- transcipient.

ance markers of the recipient strain. Although spontaneous Tc^r mutants of *B. cereus* 569 were occasionally found at low frequencies, we never observed such spontaneous mutants of *B. anthracis*.

Formation of parasporal crystals by *B. anthracis* and *B. cereus* transcipients. Phase microscopy revealed that some Tc^r transcipients derived from matings in which strain 4042A UM8 td2 was the donor had also acquired the ability to produce parasporal crystals (Cry^+) resembling those produced by the *B. thuringiensis* subsp. *thuringiensis* donor (Fig. 3). In contrast, no Cry^+ transcipients were obtained from matings in which strain 4042A UM8-13 td1-A was the donor. Plasmid analysis of the two donor strains indicated that pXO12 was associated with crystal production. The plasmid profiles of the Cry^- mutant 4042A UM8-13 td1-A and the Cry^+ mutant 4042A UM8 td2 were identical except for the absence (spontaneous loss) of pXO12 from UM8-13 td1-A (Fig. 2, lanes 1 and 6). This, along with the fact that there was a strict correlation between the Cry^+ phenotype and the presence of pXO12 in transcipients, is strong evidence that pXO12 is involved in crystal production. A number of reports have established that one or more plasmids are involved in parasporal crystal formation in a variety of *B. thuringiensis* strains (4–9, 11–13, 16, 17). Although both Cry^+ and Cry^- colonies were present among the Tc^r transcipients of *B. anthracis* and *B. cereus* obtained from matings in which strain 4042A UM8 td2 was the donor, the frequency of Cry^+ transcipients was much lower than that of Cry^- transcipients. As determined by phase microscopy, only 1 of ca. 500 Tc^r transcipients was Cry^+. The lower frequency of Cry^+ transcipients is in agreement with plasmid analyses which showed that donor strain 4042A UM8 td2 transferred pXO12 much less frequently than pXO11.

Transfer of pBC16 and other plasmids from *B. anthracis* and *B. cereus* transcipients. In our system of labeling transcipients for identification (e.g., Weybridge UM44 tr203-1), the first term (e.g., UM44) designates the recipient from which the transcipient was derived and the second term (e.g., tr203-1) identifies a particular transcipient purified by single-colony isolation. Transcipients isolated from mating mixtures in which *B. thuringiensis* subsp. *thuringiensis* was the donor are referred to as primary transcipients. Secondary transcipients are those derived from matings in which the donors were fertile *B. cereus* or *B. anthracis* transcipients harboring either pXO11 or pXO12.

Matings were performed to determine whether primary and secondary *B. anthracis* and *B. cereus* transcipients could also function as donors of pBC16 to Tc^s *B. anthracis* and *B. cereus* recipients. The results (Table 3) show that *B. anthracis* and *B. cereus* transcipients which acquired either pXO11 or pXO12 were, in turn, effective donors of pBC16. Plasmid analysis confirmed the transfer of pBC16 as well as other *B. thuringiensis* subsp. *thuringiensis* plasmids from the primary and secondary transcipients. Transcipients which inherited only the smaller *B. thuringiensis* subsp. *thuringiensis* plasmids migrating below chromosomal DNA (Fig. 1, lane 8) were not fertile. The donor ability of the fertile transcipients was stably maintained during subsequent growth and sporulation. Neither pBC16 nor pXO1 was effective in promoting plasmid transfer; this was evidenced by the fact that cells of *B. anthracis*(pXO1) or *B. cereus* into which pBC16 was introduced by transduction were completely devoid of donor activity.

Transcipients that harbored pXO12 were more effective donors of pBC16 than were those that harbored pXO11 (Table 3). The data also reflect a difference between *B. cereus* and *B. anthracis* in their activity as recipients. In experiments with $(pXO12)^+$ transcipients as donors, *B. cereus* recipients usually yielded 10- to 100-fold more transcipients than did *B. anthracis* recipients. However, with $(pXO11)^+$ transcipients as donors, recipient cells of the two

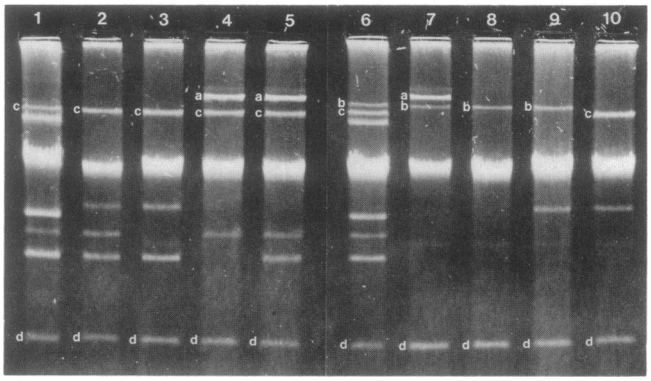

FIG. 2. Agarose gel electrophoresis of plasmid DNA from *B. thuringiensis* subsp. *thuringiensis* donor strains and *B. anthracis* and *B. cereus* transcipients. Plasmid designations are the same as in Fig. 1. Lanes: 1, *B. thuringiensis* subsp. *thuringiensis* 4042A UM8-13 td1-A, Cry^- donor; 2, *B. cereus* 569 UM20-1 tr2B-3, Cry^- transcipient; 3, *B. cereus* 569 UM20-1 tr2B-1, Cry^- transcipient; 4, *B. anthracis* Weybridge UM44-1 tr84-6, Cry^- transcipient; 5, *B. anthracis* Weybridge UM44-1 tr84-7, Cry^- transcipient; 6, *B. thuringiensis* subsp. *thuringiensis* 4042A UM8 td2, Cry^+ donor; 7, *B. anthracis* Weybridge UM44-1 tr203-23, Cry^+ transcipient; 8, *B. anthracis* Weybridge A UM23C2 tr237-10, Cry^+ transcipient; 9, *B. cereus* 569 UM20-1 tr195B-35, Cry^+ transcipient; 10, *B. cereus* 569 UM20-1 tr2B-4, Cry^- transcipient.

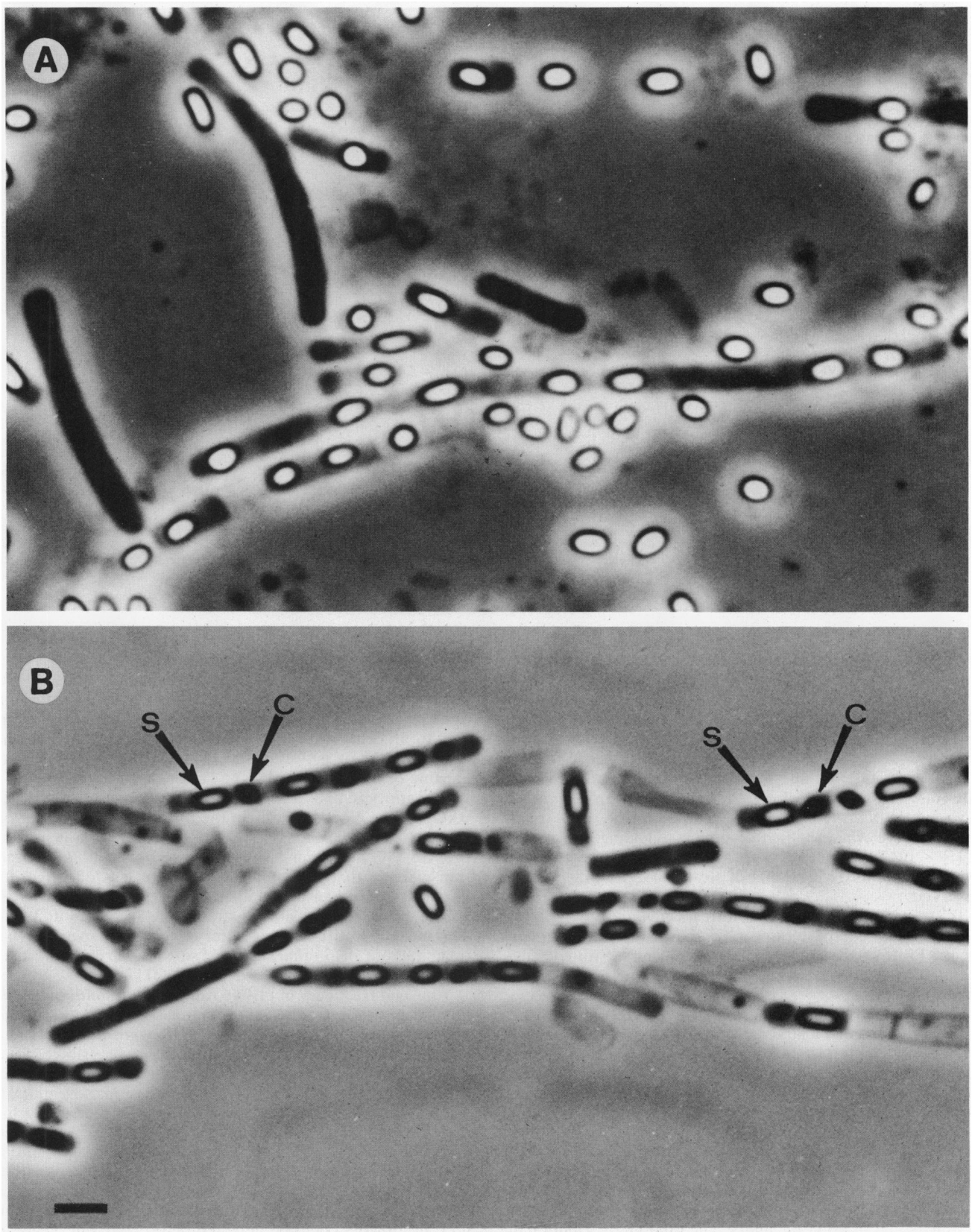

FIG. 3. Phase-contrast photomicrographs of *B. anthracis* grown at 30°C on NBY agar. (A) Strain Weybridge UM44-1. There are numerous spores (S) but no crystals (C). (B) Strain Weybridge UM44-1(pXO12). There are numerous spores and crystals. Bar, 2.0 μm.

TABLE 3. Effectiveness of *B. anthracis* and *B. cereus* transcipients as donors in the transfer of pBC16[a]

Donor strain	Tc^r transcipients with recipient strain:			
	B. anthracis		*B. cereus*	
	No. per ml	Frequency (no. per donor)	No. per ml	Frequency (no. per donor)
Primary transcipients				
B. anthracis Weybridge				
UM44-1 tr84-6(pXO1, pXO11, pBC16) Cry$^-$	4.9×10^2	1.1×10^{-5}	1.5×10^2	3.5×10^{-6}
UM44-1 tr203-1(pXO1, pXO12, pBC16) Cry$^+$	2.7×10^5	6.3×10^{-3}	1.0×10^7	2.3×10^{-1}
UM44-1 tr203-7(pXO1, pXO12, pBC16) Cry$^+$	1.3×10^5	3.0×10^{-3}	7.5×10^6	1.7×10^{-1}
UM44-1 tr203-23(pXO1, pXO12, pBC16) Cry$^+$	1.2×10^5	2.8×10^{-3}	2.6×10^7	6.0×10^{-1}
UM44-1 tr203-28(pXO1, pXO11, pBC16) Cry$^-$	5.0×10^2	1.2×10^{-5}	4.2×10^2	9.8×10^{-6}
B. cereus 569				
UM20-1 tr2B-1(pXO11, pBC16) Cry$^-$	3.5×10^1	8.1×10^{-7}	NT[b]	NT
UM20-1 tr2B-3(pXO11, pBC16) Cry$^-$	4.5×10^1	1.0×10^{-6}	NT	NT
UM20-1 tr2B-4(pXO11, pBC16)Cry$^-$	2.0×10^1	4.7×10^{-7}	NT	NT
Secondary transcipients				
B. anthracis Weybridge A				
UM17 tr57B-6(pXO1, pXO12, pBC16) Cry$^+$	1.0×10^5	2.3×10^{-3}	2.1×10^6	4.9×10^{-2}
UM23C2 tr45B-12(pXO11, pBC16) (pXO1)$^-$ Cry$^-$	6.7×10^2	1.6×10^{-5}	2.7×10^2	6.3×10^{-6}
UM23C2 tr60B-1(pXO12, pBC16) (pXO1)$^-$ Cry$^+$	8.0×10^4	1.9×10^{-3}	2.0×10^6	4.7×10^{-2}
UM23C2 tr96B-3(pXO11, pBC16) (pXO1)$^-$ Cry$^-$	8.8×10^2	2.0×10^{-5}	5.2×10^2	1.2×10^{-5}
UM23C2 tr237-10(pXO12, pBC16) (pXO1)$^-$ Cry$^+$	7.9×10^5	1.8×10^{-2}	3.5×10^7	8.1×10^{-1}
B. cereus 569				
UM20-1 tr210B-1(pXO11, pBC16) Cry$^-$	7.1×10^1	1.6×10^{-7}	3.5×10^1	8.0×10^{-8}
UM20-1 tr251-1(pXO12, pBC16) Cry$^+$	1.3×10^5	3.0×10^{-4}	8.6×10^5	2.0×10^{-3}
UM20-1 tr251-5(pBC16) Cry$^-$	0	0	0	0

[a] To permit selection and identification of Tc^r transcipients, we used *B. cereus* and *B. anthracis* recipients which had auxotrophic requirements different from those of the respective donors. We never observed any effect of auxotrophic mutations on donor or recipient ability. Mating mixtures were incubated for 20 h, at which time the average number of *B. anthracis* donors per milliliter was 4.3×10^7 CFU and that of *B. cereus* donors was 4.4×10^8 CFU. Frequency is expressed as the number of transcipients per donor. The values given are averages of results from at least two experiments.

[b] NT, Not tested.

species yielded approximately equal numbers of transcipients.

B. anthracis and *B. cereus* transcipients that contained pXO12 were not tested for transfer of pBC16 to *B. thuringiensis* subsp. *thuringiensis*. However, the primary transcipients of *B. cereus* 569 UM20-1 listed in Table 3, tr2B-1, tr2B-3, and tr2B-4, all of which contained pXO11 and pBC16, were tested as donors in matings with *B. thuringiensis* subsp. *thuringiensis* 4042A UM8-13. The three transcipients were equally effective donors, yielding an average of 1.1×10^4 Tc^r transcipients per ml (frequency, 2.3×10^{-5}). No spontaneous Tc^r mutants of strain 4042A UM8-13 were observed.

Tc^r transcipients from both intraspecies and interspecies matings retained the auxotrophic marker of the recipient strain. Prototrophic recombinants were never found, suggesting that transfer of chromosomal DNA occurred rarely or not at all.

Evidence for plasmid mobilization by pXO11 and pXO12. Taking advantage of a large collection of *B. anthracis* and *B. cereus* transcipients displaying various plasmid profiles, we attempted to identify the fertility factors responsible for plasmid mobilization. Based on the fact that all transcipients harboring either pXO11 or pXO12 were capable of plasmid transfer, we speculated that either of these two plasmids alone could confer donor capability to host cells. Examination of the plasmid content and transfer ability of numerous Tc^r transcipients confirmed that both pXO11 and pXO12 are fertility plasmids, each capable of promoting its own transfer as well as that of other plasmids. The random distribution of the smaller *B. thuringiensis* subsp. *thuringiensis* plasmids in fertile transcipients suggested that no combination of these plasmids in conjunction with either pXO11 or pXO12 was necessary for plasmid transfer. Furthermore, transcipients acquiring only these small *B. thuringiensis* subsp. *thuringiensis* plasmids (Fig. 1, lane 8) were ineffective in transferring pBC16. In contrast, transcipients inheriting only pBC16 and either pXO11 or pXO12 (Fig. 2, lanes 7 through 10) were capable of transferring pBC16 (Table 3).

B. anthracis and *B. cereus* transcipients that harbored pXO12 were more fertile than the original donor, *B. thuringiensis* subsp. *thuringiensis* 4042A UM8 td2, in transferring pBC16. On the other hand, *B. anthracis* and *B. cereus* transcipients that inherited pXO11 were less effective donors of pBC16 than were either of the two *B. thuringiensis* subsp. *thuringiensis* donor strains 4042A UM8 td2 and 4042A UM8-13 td1-A. This latter observation suggests that other factors in *B. thuringiensis* subsp. *thuringiensis* may contribute to the donor activity of pXO11.

Time and frequency of pBC16 transfer by *B. anthracis*. The number of transcipients from a mating between a *B. anthracis* donor carrying pXO12 and a *B. anthracis* recipient increased rapidly between 2 and 6 h and very slowly after that (Fig. 4). The greatest relative increase (164-fold) in transcipients occurred between 2 and 4 h of mating, indicating that many independent transfer events occurred during that period. Comparable results were obtained with *B. anthracis* donors carrying pXO11 and with *B. cereus* and *B. thuringiensis* subsp. *thuringiensis* donors carrying either pXO11 or pXO12 (data not shown). In experiments in which mating mixtures were sampled at 0, 30, 60, 90, and 120 min, no transcipients could be detected before 120 min, suggesting that a period for donor and recipient cells to grow together was required before plasmid transfer could occur.

The necessity for exponential growth of donor and recipient cells together was further supported by the failure to detect transcipients in mating mixtures prepared from donors and recipients which had been grown separately for increasing periods of time (4 to 16 h) before they were mixed.

Mechanism of transfer. To investigate the possibility of phage-mediated plasmid transfer, we tested cell-free filtrates prepared from donor cultures for the ability to convert recipients to tetracycline resistance. No Tc^r transcipients could be detected in such experiments. To determine whether plasmid transfer occurred by transformation, we examined the sensitivity of pBC16 transfer to DNase as described above. In matings between *B. anthracis* donors and *B. cereus* recipients, the number of Tc^r transcipients obtained after 4 h in the presence of DNase and $MgSO_4$ (3.2×10^6 per ml) was not significantly different from the number obtained in the presence of $MgSO_4$ alone (3.0×10^6 per ml). Finally, to determine whether cell-to-cell contact was necessary for plasmid transfer, we conducted a mating between a *B. anthracis* donor and a *B. cereus* recipient in a U tube. A 0.45-μm filter (type HA; Millipore) inserted between the two strains prevented cell-to-cell contact but allowed diffusion of filterable material between the two cultures. As a control, the two cultures were also mixed together in a second U tube without a filter. After 3 h of incubation, normal numbers of transcipients were recovered from the tube without the filter, but no transcipients were detected in samples from the tube containing the filter.

DISCUSSION

The results presented here demonstrate that each of two plasmids, pXO11 and pXO12, indigenous to strain 4042A of *B. thuringiensis* subsp. *thuringiensis* is capable of promoting plasmid transfer within and among strains of *B. anthracis*, *B. cereus*, and *B. thuringiensis*. All transfer-proficient *B. anthracis* and *B. cereus* transcipients thus far examined inherited either pXO11 or pXO12. That pXO11 and pXO12 can function independently of the low-molecular-weight plasmids of *B. thuringiensis* subsp. *thuringiensis* is direct proof that they are fertility plasmids capable of bringing about their own transfer as well as that of other plasmids. Transcipients harboring pXO12 were more effective donors than those harboring pXO11, and *B. cereus* was generally a better recipient than *B. anthracis*. For example, the maximum frequency for pBC16 transfer by $(pXO12)^+$ *B. anthracis* donors to *B. anthracis* and *B. cereus* recipients was 5 and 80%, respectively.

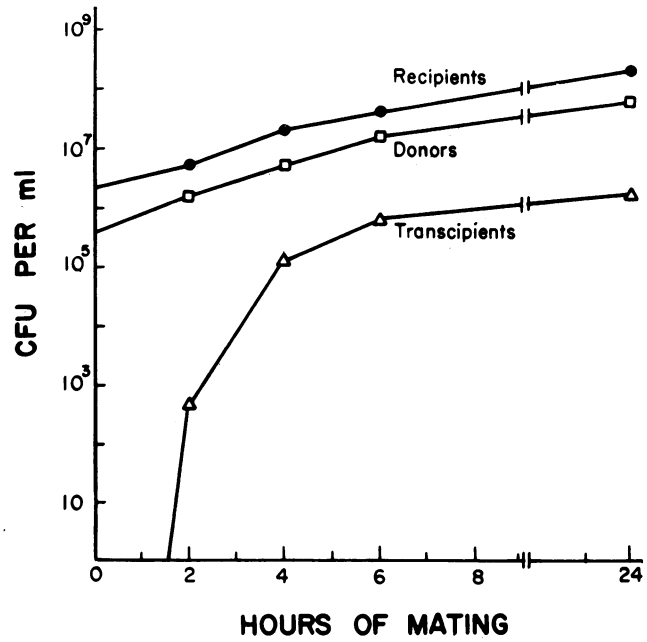

FIG. 4. Transfer of pBC16 from *B. anthracis* Weybridge A UM23C2 tr237-10(pX012, pBC16) Ura^- Tc^r Cry^+ to *B. anthracis* Weybridge UM44-1 Ind^- Str^r. At the indicated times, samples were plated on L agar containing tetracycline or streptomycin or both to score donors, recipients, and transcipients, respectively.

Plasmid pXO12 was inherited less frequently than pXO11 by recipients mated with the *B. thuringiensis* subsp. *thuringiensis* donor which carried both pXO11 and pXO12. However, once the two plasmids were segregated, transcipients inheriting pXO12 were more fertile than those inheriting pXO11. These observations, together with the failure to detect transcipients that had acquired both pXO11 and pXO12, suggest that these two fertility plasmids may compete in the transfer process. An analogous competition phenomenon has been reported by Clewell (3) for streptococci matings in which the transfer ability of the conjugative erythromycin resistance plasmid pAMβ1 is drastically reduced in the presence of either of two other conjugative plasmids, pAMγ1 and pAD1.

Although the mechanism of transfer is still unknown, several lines of evidence support a conjugation-like process: (i) the addition of DNase to mating mixtures did not reduce transfer frequencies; (ii) donor filtrates were inactive and cell-to-cell contact was necessary; (iii) the high frequencies of transfer are typical of conjugation systems; and (iv) the large increase in the number of transcipients between 2 and 4 h (10^2- to 10^5-fold) indicates that many independent transfer events occurred. Our results, showing a requirement for cell-to-cell contact and the ineffectiveness of DNase in preventing plasmid transfer from *B. anthracis* donors, are similar to those obtained by Gonzalez and Carlton (6) in plasmid transfer experiments with *B. thuringiensis* donors.

There appeared to be an essential period (2 to 4 h) for growth of donor and recipient cells together before plasmid transfer could be detected. The requirement for growing donor and recipient cells together during the exponential phase of growth was dramatically illustrated by the drastic reduction in plasmid transfer when mating mixtures were prepared from donor and recipient cells grown separately for similar periods of time.

In addition to transfer functions, the fertility plasmid pXO12 was found to carry information involved in parasporal crystal formation, which was expressed in all three species of *Bacillus* tested. The evidence for this was twofold. (i) All transcipients harboring pXO12 were Cry^+, whereas those harboring pXO11 were Cry^-, and (ii) the inability of strain 4042A UM8-13 td1-A to produce parasporal crystals when infected with the converting phage TP-13 (see Table 1, footnote *g*) was correlated with the spontaneous loss of pXO12.

The ability to transfer a large range of plasmids makes this a useful genetic exchange system for the functional analysis of genetic determinants on plasmids of *B. anthracis*, *B. cereus*, and *B. thuringiensis*. For example, we have used this mating system to transfer the *B. anthracis* plasmids pXO1 (C. B. Thorne, *in* L. Leive, ed., *Microbiology—1985*, in press) and pXO2 (B. D. Green, L. Battisti, and C. B. Thorne, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, H99,

p. 124), which are involved in the synthesis of anthrax toxin and capsules, respectively, to *B. cereus* and strains of *B. anthracis* previously cured of the plasmids.

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